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Review

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Omics Approaches To Probe Microbiota and Drug Metabolism Interactions

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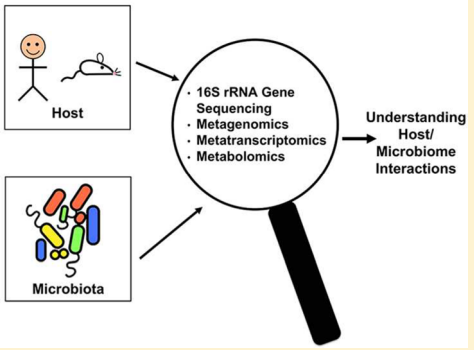
ABSTRACT: The drug metabolism field has long recognized the beneficial and sometimes deleterious influence of microbiota in the absorption, distribution, metabolism, and excretion of drugs. Early pioneering work with the sulfanilamide precursor prontosil pointed toward the necessity not only to better understand the metabolic capabilities of the microbiota but also, importantly, to identify the specific microbiota involved in the generation and metabolism of drugs. However, technological limitations important for cataloging the microbiota community as well as for understanding and/or predicting their metabolic capabilities hindered progress. Current advances including mass spectrometry-based metabolite profiling as well as culture-independent sequence-based identification and functional analysis of microbiota have begun to shed light on microbial metabolism. In this review, case studies will be presented to highlight key aspects (e.g., microbiota identification, metabolic function and prediction, metabolite identification, and profiling) that have helped to clarify how the microbiota might impact or be impacted by drug metabolism. Lastly, a perspective of the future of this field is presented that takes into account what important knowledge is lacking and how to tackle these problems.



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1. INTRODUCTION

Bacteria living on and within our bodies make important metabolic contributions, whether it is extracting calories from the diet[1] or contributing to the metabolism of drugs.[2] While this review uses examples from bacteria involved in modulating (or being modulated by) drug metabolism in animal models and humans, many of the concepts can be applied to bacteria in symbiotic relationships or those in the environment that may encounter drugs or toxicants through deliberate application (e.g., pesticides) or through contamination (e.g., persistent organic pollutants, antibiotics, and other drugs in wastewater). For example, a symbiotic and mutualistic relationship[3] was recently clarified between the coffee berry borer (*Hypothenemus hampei*) and *Pseodomans fulva*, an important bacteria in the borer gut that permits it to survive in a caffeine-rich environment. Similarly, the bean bug, *Riptortus pedestris*, can degrade fenitrothion (an insecticide) through the metabolic activity of *Burkholderia* spp. in its gut.[4] As will be discussed later, cutting-edge tools including 16S rRNA gene-based microbial community profiling, functional prediction, and mass spectrometry-based metabolite profiling were instrumental in identifying how these relationships benefit the host and the gut microbe.

From the discovery of prontosil as a precursor to the microbial-derived antibiotic sulfanilamide[5,6] to a more recent example with the cardiac glycoside digoxin,[7] research has only just scratched the surface of the incredibly complex and vital role that the microbiota can play both in terms of their contribution to pathologic conditions as well as serving as potential targets of (or sources of) therapeutic intervention. More than 50 years ago[8−10] it was appreciated that the microbiota could perform biotransformation including hydrolysis, oxidation, reduction, and β-glucuronidase reactions and that drugs could also modulate the microbiota structure. For

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example, the microbiota substantially influences the metabolism of L-DOPA (levodopa, L-3,4-dihydroxyphenylalanine).[11] It was reported that following L-DOPA feeding to germ-free mice *m*-hydroxyphenylacetic acid (*m*-HPAA) was not detected when urine extracts were analyzed via gas chromatography because the microbiota are needed to convert L-DOPA to *m*-tyramine, which is then converted to *m*-HPAA.[11] Thus, reduced metabolism of L-DOPA in germ-free mice greatly prolongs L-DOPA half-life and hence activity. This observation and others[12] are important when considering how the gut microbiota may promote adverse drug effects. Similarly, lactulose, a common treatment for constipation, was found to require several bacterial strains including *Bacteroides* and *Bifidobacterium* to metabolize lactulose into its therapeutic metabolites of lactic acid and acetic acid.[13] An extensive table of drugs modulated by the microbiota has been reported elsewhere.[14]

A more recent example of how the gut microbiota influences drug metabolism is through studies of the anticancer drug irinotecan (CPT-11).[15,16] Gut bacteria can "re-activate" glucuronidated drugs through their β-glucuronidase enzyme activity, and in the case of irinotecan, this process causes gut inflammation and irritation.[15,16] The authors reported that partnering irinotecan with a β-glucuronidase inhibitor while not influencing serum levels of the activated drug decreased the amount of harmful metabolites produced from the bacteria, thus alleviating some of the gastrointestinal inflammation.[16] This example also highlights the relationship between the gut microbiota and enterohepatic circulation, a process that has been recognized for decades.[17] Bacterial β-glucuronidases are reported to be primary causes of nonsteroidal anti-inflammatory drugs (NSAID)-derived enteropathy.[18] Similarly to irinotecan, glucuronidated NSAIDs are transported into the intestine via enterohepatic circulation where gut bacteria (in this[18] example, *Escherichia coli*) will cleave the glucuronide, thus yielding harmful metabolites that cause the formation of ulcers in the gut.

Technology developments have significantly accelerated progress in the area of microbial-mediated drug metabolism. In order to illustrate how technology has greatly enhanced the discovery process with respect to microbiota-mediated drug metabolism, we highlight two examples: sodium cyclamate studies from the 1960s and recent studies with the cardiac glycoside digoxin.

Sodium cyclamate (sodium *N*-cyclohexanesulfamate) is an artificial sweetener that was shown to be metabolized to the potential human carcinogen cyclohexylamine, thus leading the FDA to ban it in 1970. Initial work by Kojima and Ichibagase[19] identified the conversion of sodium cyclamate to cyclohexylamine, and later work suggested that this process was mediated by microbiota inhabiting the gastrointestinal tract.[20,21] Here, only one volunteer (out of three total) fed a diet rich in sodium cyclamate produced high levels of cylohexylamine, demonstrating how gut microbiota contribute extensively to the metabolic phenotype or metabotype.[22] Furthermore, the production of cyclohexylamine was dose-dependent.[23] Other reports demonstrated that rats could transfer the cyclohexylamine-producing capability to one another, likely through coprophagy, and that antibiotic treatment could dramatically reduce cyclohexylamine production.[24] Culture-based methods suggested that members of the *Clostridia* class were likely involved in the metabolic conversion in rats.[21] It was also suggested that *Enterococci* could be responsible for the conversion from cyclamate to cyclohexylamine in humans.[20,21] Along with the bacterial identification, two possible mechanisms were proposed. The first is a simple hydrolysis to remove the hydrogen sulfate ion, and the second is a hydrogen reduction to remove a bisulfite ion. Both mechanisms by *Enterococci* can yield cyclohexylamine. However, as was suggested by Scheline,[10] many of these culture-based methods not only were limited in terms of what could be cultured but also were likely misleading given the inability to accurately identify the bacteria. Much of the metabolism work with sodium cyclamate was made possible by tracing the fate of ^{14}C-labeled sodium cyclamate from tissues or cecal/fecal incubations using paper chromatography, radiochromatogram scanning, or gas chromatography coupled with chemical ionization mass spectrometry (GC-MS),[25,26] all relatively sophisticated techniques at the time. Furthermore, Williams[27] lamented that metabolite identification studies were not only crucial to understanding the metabolic fate of drugs but also laborious and in some cases extremely challenging given the limited analytical chemistry resources available to investigators at the time.

Digoxin is a cardiac glycoside that is used worldwide for the treatment of atrial fibrillation and congenital heart failure. Its primary mechanism of action is through inhibition of Na−K-ATPase membrane pumps, thus increasing intracellular sodium levels; by means of compensation, the sodium calcium exchanger increases intracellular calcium to offset this effect. The result therefore is to increase cardiac output and systolic blood pressure as well as to slow the heart rate. Early observations[28] with digoxin suggested that indeed it too was metabolized by a member of the gut microbiota, *Eggerthella lenta* (previously *Eubacterium lenta*), to produce a metabolite that lacks cardiac activity (e.g., dihydrodigoxin, a digoxin reduction metabolite). Digoxin inactivation by gut microbiota was initially speculated and later confirmed in a series of elegant papers implicating *E. lenta* as the microbe responsible for generating the cardioinactive metabolites.[29] However, the mechanism by which digoxin is converted to dihydrodigoxin and other reduction products has only recently been established[7] and importantly raises the possibility that patients might be identified based on their microbial populations as either potential responders or nonresponders to digoxin therapy. Haiser et al.[7] identified that the *cgr* operon within certain strains of *E. lenta* is induced and responsible for the reduction of digoxin using sophisticated RNA-seq analysis of microbiota gene expression. The authors proposed that this operon is intended to reduce fumarate in *E. lenta*; however, the unsaturated lactone present on the end of digoxin can also be used as a substrate for this operon. This results in saturation of the lactone, thus inactivating digoxin in the body. Furthermore, they used combinations of liquid chromatography coupled with mass spectrometry (LC-MS) and mutliple reaction monitoring (MRM) to carefully identify and quantitate the production of digoxin metabolites, capabilities that were simply not yet available to the cyclamate investigators (the triple quadrupole mass spectrometer was developed in 1978 by Enke and Yost of Michigan State University[30,31]). The ease of use and increased senstivity of the new LC-MS/MS systems would have greatly hastened the study of sodium cyclamate metabolism.

In general, it is the combined use of sequence-based approaches as well as the functional, metabolic readouts provided by mass spectrometry-based metabolite profiling and metabolomics that have helped to reinvigorate the field of microbiota-mediated drug metabolism and accelerated

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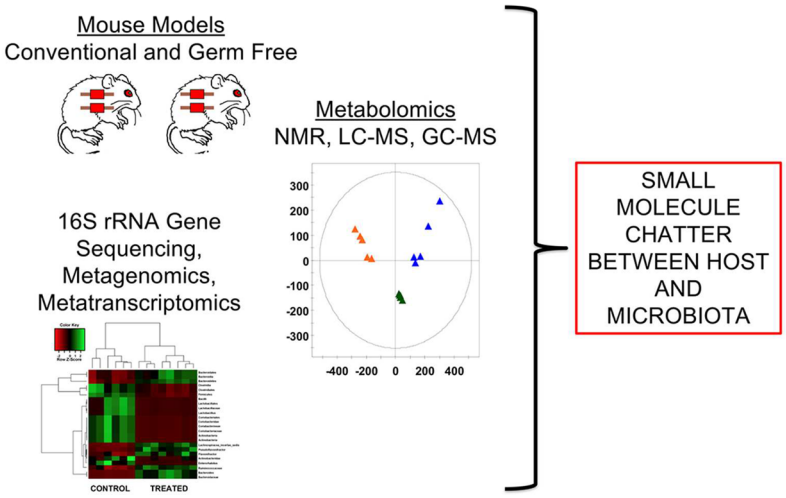

Figure 1. Metabolites produced or modified by the gut microbiota (i.e., metabolic chatter) can be studied using a variety of models and technological approaches. The contribution of the gut microbiota can be studied through comparisons of conventional and germ-free mice. Analyses of the gut microbiota can be achieved through culture-independent sequencing approaches like 16S rRNA gene sequencing, metagenomics, and/or metatranscriptomics. Reflections of the changes in gut microbial community structure and function as well as changes in host metabolism can be monitored by metabolomics.

discoveries in this space. Further unique mouse models including those lacking microbiota (herein referred to as germ-free mice) have provided new perspectives of the drug metabolism process[32−34] and will likely urge not only the drug metabolism experts but also toxicologists to reconsider the impact of the microbiota on efficacy, potency, and toxicity.[35] In the following section, we discuss the concept of microbial metabolic chatter (Figure 1) and the essential tools required to listen in and decode these signals.

2. DETECTING AND DECODING MICROBIAL CHATTER

Small molecule "microbial chatter" can be used to describe how bacteria communicate with each other and with the host. For example, signaling networks based on quorum sensing molecules[36] can help a bacterial population sense its size as well as to prevent or limit the growth of competing populations. Microbial-generated metabolites or host metabolites acted upon by microbial metabolism (i.e., cometabolites) can interact with the host in a similar manner. Hence, the concept of small molecule "microbial chatter" represents a means by which the host and microbiota communicate, and this signaling network has just started to be unraveled through the application of mass spectrometry-based profiling and sequencing-based methods described below.

2.1. Signatures of Microbial Metabolism and Composition. Metabolomics can simply be defined as the process of identifying chemical fingerprints left behind by biological processes. Initially, the phrase "biological processes" most exclusively referred to those generated by the host, but numerous examples throughout the metabolomic literature suggest that microbiota-generated metabolites[37−39] or host metabolites modified[40−43] by the microbiota (also known as cometabolites) significantly "contaminate" the metabolomic signature. These observations have provided strong support for the use of metabolomics in the investigation of the microbiome. For example, 2,8-dihydroxyquinoline (2,8-DHQ) and its glucuronide (2,8-DHQ-β-D-Gluc) were found to be enriched (Table 1) in the urine of mice treated with the peroxisome proliferator-activated receptor alpha (PPARα) agonist WY-14,643.[44] Mice treated with the antioxidant tempol similarly have increased excretion of 2,8-DHQ and 2,8-DHQ-β-D-Gluc in their urine.[39] While never confirmed specifically in these studies to be of bacterial origin, Wikoff et al.[37] reported in a mass spectrometry-based metabolomics investigation of conventional (those with microbiota) and germ-free mice that DHQ production was highly dependent on the presence of bacteria. Others have reported that isomers of 2,8-DHQ, including 2,4-DHQ, are important quorum sensing molecules that may be involved in the pathogenicity of *Pseudomonas aeruginosa*, although it remains to be determined if 2,8-DHQ plays a similar quorum sensing role or which microbe is generating it.

Like 2,8-DHQ, other metabolites (Table 1) may likely serve as important indicators of changes in the composition and/or metabolic activity of the microbiota. For example, hippuric acid, the glycine conjugate of benzoic acid, has a strong connection with the gut microbiota, and its concentration in urine has been shown to be dramatically reduced in either germ-free mice or in mice treated with antibiotics.[45] *p*-Cresol, trimethylamine, trimethylamine *N*-oxide, and short chain fatty acids are similarly known to be produced by microbiota, and their concentrations can be perturbed following drug treatment (Table 1). A targeted profiling study[46] of urine collected from INTERMAP (**INTER**national collaborative study of **MA**cronutrients, micronutrients, and blood **P**ressure) examined the gut microbial metabolites phenylacetylglutamine, 3-cresol sulfate, and hippuric acid in 4000 samples and identified unique signatures associated with age, race, and gender. The authors conclude that such approaches based on LC-MS/MS may serve as a rapid and convenient way to ascertain the physiologic state of the gut microbiota under various conditions. However, despite our capabilities of measuring their concentrations by LC-MS/MS, the identities of the bacteria responsible for generating these metabolites remain unknown.

Recent studies of bile acids are emblematic of how mass spectrometry and sequencing-based approaches of the microbiota can be instrumental in advancing our understanding of how metabolites signal between the host and the gut

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Table 1. Metabolites Modified or Produced by Gut Microbiota

metabolite	treatment	source	host	mechanism	significance	method	ref
TβMCA[a]	Tempol (antioxidant)	Intestinal tissue	Mice	Tempol reduces BSH[b] activity of *Lactobacillus*, causing an accumulation of TβMCA[a] and inhibition of FXR[c] signaling, producing antiobesity effects	Potential target of antiobesity drugs	UHPLC-ESI-QTOFMS[d]	Fei Li, *Nature Communications*, 2013[38]
Hippuric acid	Rifaximin (PXR[e] agonist)	Intestinal tissue	Mice	Mice treated with antibiotic rifaximin showed decrease in microbial metabolites such as hippuric acid	Influence of gut microbiota on absorption and distribution of naringenin	HPLC-ESI-MS/MS[f]	Naiara Orrego-Lagaron, *British Journal of Nutrition*, 2015[90]
	Wy-14643[g] (PPARα[h] agonist)	Urine	Mice	PPARα[h] agonist increased glycine conjugation of benzoic acid to hippuric acid	Urinary biomarkers of PPARα[h] activation as a result of gut microbiota	UHPLC-TOFMS[i]	Yueying Zhen, Molecular Endocrinology, 2009[44]
p-cresol sulfate	Rifampin (PXR[e] agonist)	Urine	Humans	The bacterial metabolite of taurine, *p*-cresol, decreased with antibiotic rifampin administration	Potential drug−drug interactions of PXR[e] activators	UHPLC-TOFMS[i]; GC-MS[j]	Bora Kim, *Journal of Proteome Research*, 2013[91]
	Taurine (amino sulfonic acid)	Urine	Rats	Taurine supplementation caused an increased output of *p*-cresol sulfate, decreasing blood pressure in spontaneously hypertensive rats	Preventative effects in cardiovascular disease	^{1}H NMR[k]	Kazuki Akira, *Journal of Pharmaceutical and Biomedical Analysis*, 2013[92]
	Acetaminophen (antipyretic)	Urine	Humans	Competitive O-sulfonation of p-cresol reduces capacity to sulfonate acetaminophen	Influence of gut bacteria on drug-induced responses involving sulfonation	^{1}H NMR[k]	T. Andrew Clayton, *Proceedings of the National Academy of Sciences*, 2009[93]
TMA[l], TMAO[m]	3,3-dimethyl-1-butanol (inhibits TMA[l] lyase)	Liver, kidney, plasma, urine	Mice	3,3-dimethyl-1-butanol inhibition of TMA[l] lyase inhibits microbial production of TMA[l], reducing TMAO[m] levels	Potential treatment of atherosclerosis and other cardiometabolic diseases	LC-MS/MS[n]	Zeneng Wang, *Cell*, 2015[94]
	L-carnitine (amino acid derivative)	Plasma	Humans	Oral L-carnitine is metabolized to TMA[l] by intestinal microbiota. Further metabolism produces TMAO[m]. L-carnitine also decreases AGE[o] levels	Protective properties on vascular injuries in human patients on hemodialysis	GC[p]	Kei Fukami, *Journal of Cardiovascular Pharmacology*, 2015[95]
SCFA[q]	Oxidized ethyl-linoleate (induces mucosal hypertrophy)	Intestinal tissue	Rats	Oxidized ethyl linoleate decreases cecal fermentation of dietary fiber, changing SCFA[q] composition	SCFA[q] potential inhibition of growth or proliferation of tumor cells	GC[p]	Hiroshi Hara, *Journal of Nutrition*, 1996[96]
	Sericin (antioxidant)	Feces	Rats	Dietary sericin elevates cecal production of organic acids such as acetate and *n*-butyrate in rats fed a high-fat diet	Sericin use as a prebiotic to improve colon health	GC[p]	Yukako Okazaki, *Journal of Nutrition*, 2011[97]
SCFA[q] (acetate)	B-GOS[r] (prebiotic oligosaccharide)	Feces	Humans	Fermentation analysis of B-GOS[r] *in vitro* revealed increases in acetate production correlated with increase in *Bifidobacterium* populations	Potential use of B-GOS[r] as a prebiotic to modulate gut microbiota poplation	GC[p]	Roberta Grimaldi, *British Journal of Nutrition*, 2016[98]
SCFA[q] (propionate)	Aspartame (artificial, nonsaccharide sweetener)	Serum	Rats	Aspartame increased total bacteria, *Enterobacteriaceae*, and *Clostridium leptum*. Aspartame was rapidly metabolized and associated with elevations in propionate	Aspartame involvement in increased risk of metabolic diseases	^{1}H NMR[k]	Marie Palmnäs, *PLoS One*, 2014[99]
SCFA[q]; phenol, *p*-cresol	Arabinoxylan-oligosaccharides (prebiotic)	Intestinal tissue	Humans	In vitro AXOS[s] supplementation resulted in decreased production of toxic microbial degradation products, increased production of beneficial SCFA[q], and long-term effect on colonic microbe community	Use of AXOS[s] as a prebiotic to modulate microbial metabolism in distal colon	GC[p]; HPLC[t]	Juan I. Sanchez, *Microbial Biotechnology*, 2009[100]
2,8-dihydroxyquinoline	Tempol (antioxidant)	Urine	Mice	Tempol increased urinary excretion of 2,8-dihydroxyquinoline along with bacterially produced glucuronide metabolites	Tempol affects microbiota composition and microbial metabolism	LC-MS/MS[n]	Fei Li, *Journal of Proteome Research*, 2013[39]
	Wy-14643[g] (PPARα[h] agonist)	Urine	Mice	Activation of PPARα[h] increased excretion of 2,8-dihydroxyquinoline	Urinary biomarkers of PPARα[h] activation as a result of gut microbiota	UHPLC-TOFMS[i]	Yueying Zhen, *Molecular Endocrinology*, 2009[44]

[a]Taurine β muricholic acid. [b]Bile salt hydrolase. [c]Farnesoid X receptor. [d]Ultra-high pressure liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry. [e]Pregnane X receptor. [f]High-performance liquid chromatography electrospray ionization tandem mass spectrometry. [g]Pirinixic Acid. [h]Peroxisome proliferator activated receptor alpha. [i]Ultra-high pressure liquid chromatography time-of-flight mass spectrometry. [j]Gas chromatography mass spectrometry. [k]Proton nuclear magnetic resonance. [l]Trimethylamine. [m]Trimethylamine *N*-oxide. [n]Liquid chromatography tandem mass spectrometry. [o]Advanced glycation end products. [p]Gas chromatography. [q]Short chain fatty acid. [r]Bimuno-galactooligosaccharides. [s]Arabinoxylan-oligosaccharides. [t]High-pressure liquid chromatography.

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microbiota.[38,41,42] Once thought simply as detergents to help solubilize fat and fat-soluble vitamins, our appreciation of the complex and varied signaling roles that bile acids can play has grown in large part due to mass spectrometry-based characterization of bile acid pools.[47] For example, a recent report based on a combination of ultra-high-pressure liquid chromatography (UHPLC) coupled with quadrupole time-of-flight mass spectrometry (QTOFMS) for broad profiling and targeted applications using UHPLC-MS/MS have identified 145 primary, secondary, and tertiary bile acid species.[48] It should be clearly noted that in the case of bile acids where there exist numerous positional and stereochemical isomers the strategic application of UHPLC has been instrumental in helping to chromatographically resolve the stereoisomers and thus permit accurate quantitation. The extensive and sensitive capabilities afforded by the latest mass spectrometry platforms have helped to accelerate our understanding for the varied roles that bile acids can play. For example, recent observations involving mice treated with antibiotics or the antioxidant tempol suggest that perturbing the microbiota can profoundly influence the gut microbiota community structure as well as its metabolic function.[38,39,41,42,49] In these cases, the end result is a shift in the bile acid pool toward taurine conjugated bile acids (taurine conjugation predominates in mice), which in turn antagonize the farnesoid X receptor, a critical ligand activated nuclear receptor that regulates bile acid, glucose, and lipid metabolism.[50] Other examples of drugs that modulate or interact with the microbiota include the diabetes drug metformin[51] and HMG CoA reductase inhibitor simvastatin.[52]

It is important to note that in addition to mass spectrometry, sequence-based analysis of the microbiota not only is helping to elucidate how the microbiota are capable of metabolizing drugs and endogenous metabolites but also has been instrumental in identifying which bacteria are key to the process. In a recent report, Devlin and Fischbach elegantly describe a new biosynthetic pathway for bile acids modulated by the microbiota.[53] They identify a process by which gut microbiota including *E. lenta* and *Rumincoccus gnavus* can detoxify bile acids such as deoxycholic acid to their iso forms (3β-hydroxy epimer), which they report are less cytotoxic to the bacteria, although differential effects on the host were not explored. Using computational approaches, they identified the nicotinamide-dependent 3α- and 3β-hydroxysteroid dehydrogenase genes in *E. lenta* and *R. gnavus*. This approach along with classic toxicity assays and enzyme kinetics highlights the value of sequence-based approaches in discovery efforts to better understand the metabolism associated with the microbiota.

2.2. Mass Spectrometry-Based Metabolite Identification and Metabolomics. One of the most critical processes in untargeted metabolomics studies is the identification of metabolites, which is a prerequisite to relating the quantitative metabolomics data to its underlying biochemical role. Nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{13}C) and MS coupled with either liquid or gas chromatography are the techniques most commonly used for the structural determination of metabolites. NMR offers several unique advantages, but it is hampered by an overall lack of sensitivity, as well as difficulties in working with complex multicomponent mixtures. GC-MS is still the most commonly used method for the identification of unknown compounds, but the advent of electrospray ionization and atmospheric pressure chemical ionization has resulted in LC-MS playing an increasingly important role in the identification and quantitation of metabolites. Others have expertly reviewed the strengths and weakness of these platforms.[22,54−56] High-resolution accurate mass spectrometry can provide elemental formulas for the intact molecule, but fragmentation of the intact molecule either in the ionization source or a combination of collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) is necessary to begin characterization of the structure of the precursor ion.[57] Comparison of the experimentally obtained product ion mass spectra with those in metabolite databases such as METLIN,[58] MassBank,[59] the Human Metabolome Database,[60,61] and Lipid Maps[62] is typically the first step in metabolite identification. An alternative and complementary approach is to tentatively identify the metabolites by interpretation of their product ion mass spectra, which is growing in popularity as new bioinformatic and visualizations tools are being developed. There are many examples of certain classes of metabolites having diagnostic fragment ions (e.g., m/z 184 for phosphatidylcholines, lyso-phosphatidylcholines, and sphingomyelins; m/z 241 for phosphatidylinositols; m/z 85 for acylcarnitines; m/z 97 for glucosinolates) or class characteristic neutral losses (e.g., losses of 141 Da for phosphatidylethanolamines, 87 Da for phosphatidylserines, and 176 Da for glucuronides), and these are helpful in determining tentative structures. Unfortunately, many compounds do not have readily recognizable diagnostic fragment ions, and the de novo identification of metabolites by interpretation of their product ion mass spectra is extremely time-consuming and challenging.[63] Furthermore, the extensive metabolic repertoire and diverse population of the microbiota further complicate metabolite discovery efforts.

Multistage mass spectrometry (MS^n) afforded by ion trap platforms is a powerful technique for assisting in the determination of structures from interpretation of mass spectral data, especially since the advent of precursor ion fingerprinting.[64,65] Precursor ion fingerprinting is based on the premise that structurally similar compounds possess matching substructures, and these matching substructures produce matching product ion spectra. It relies on the probability of various possible decomposition products of an ion being independent of the ionization method, structure of the precursor ion, and the formation mechanism of the ion undergoing decomposition. Matching spectra of identical product ions can be acquired regardless of the original structure and MS^n stage.

Multistage Elemental Formula generator is a recently developed software package that uses the precursor product ion relations to extract the relevant data from multistage mass spectra.[64] It can be used in combination with high-resolution accurate MS^n to permit the unambiguous assignment of elemental formulas to fragment ions and to neutral losses, thereby facilitating the metabolite identification process. This fragmentation tree approach for the structural characterization of metabolites was used to successfully discriminate between the two isomeric prostaglandins PGE2 and PGD2 with 12 and 18 unique elemental formula paths, respectively.[64]

Using a combination of the above analytical approaches, it is becoming more feasible to detect, identify, and quantitate metabolites that are derived or modified from the gut microbiota. Understanding the "metabolic chatter" of the gut microbiota can inform about disease progression and provide a better understanding for how modulating the gut microbiota can significantly impact the metabolome. Data analysis and database tools including the recently developed platform MS-

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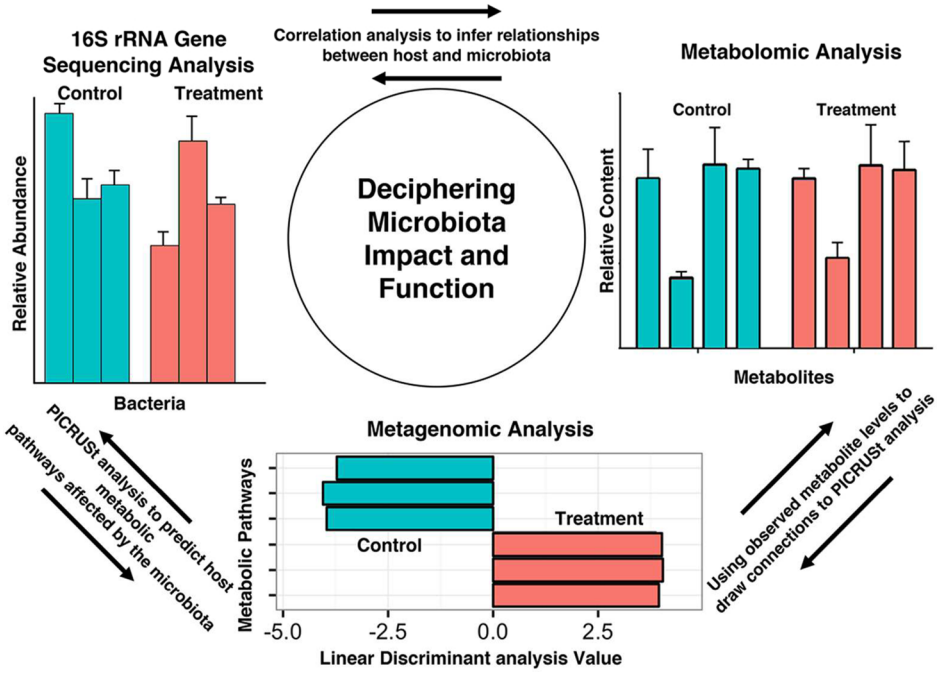

Figure 2. Understanding the complete contribution of the microbiome to drug metabolism as well as human health and disease can best be achieved through a combination of analytical techniques. 16S rRNA gene sequencing-based community analysis reveals the taxonomic profile of the gut and can be used to predict which metabolic pathways are modulated with the approaches including PICRUSt. Metabolomic analysis and carefully measured metabolite concentrations can be used to validate the pathways seen in PICRUSt. Relationships can then be drawn between specific bacterial changes and specific metabolite levels to better understand the metabolic contribution of the microbiota.

DIAL[66] will likely have a great impact on the identification of known metabolites but also will help with the identification of unknowns (using approaches like fragmentation trees described above). Short chain fatty acids (SCFAs) including butyric acid, acetic acid, and propionic acid (and other microbial cometabolites) have been a central focus with respect to microbial metabolic activity.[67] For example, it has been reported that colonocytes rely on butyric acid as an important nutrient source or that butyric acid can impact immune homeostasis.[67] Additionally, the gut microbiome can contribute to choline degradation. Sulfur reducing bacterial species like those in the class *Clostridia* can degrade choline into trimethylamine (TMA).[68] TMA can be further metabolized by hepatic flavin monooxygenase (FMO) to trimethylamine-*N*-oxide (TMAO).[69] TMAO has recently been seen to have a direct relationship to cardiovascular disease and atherosclerosis. These examples highlight the interplay between the microbiome and the metabolome, thus demonstrating their importance for understanding disease progression.

2.3. Culture-Independent Sequencing-Based Analysis and Functional Prediction. Predicting the function of the microbiota (beyond standard census taking with 16S rRNA community profiling) is perhaps the ultimate objective in microbiome research. PICRUSt (**P**hylogenic **I**nvestigation of **C**ommunities by **R**econstruction of **U**nobserved **St**ates) created by the Huttenhower lab in Harvard[70] is a bioinformatic application that infers metabolic information from 16S rRNA gene sequencing data. PICRUSt uses phylogenic trees created during 16S rRNA gene sequencing analysis to assign functional pathways to clades of bacteria.[70] A significant concern with this approach is lateral gene transfer, meaning that distantly related bacteria can share functionality, thus contradicting the idea that only bacterial clades share functionality.[70] The Huttenhower group accounted for this by incorporating ancestral state reconstruction algorithms that take into account the probability for initiation and reception of lateral gene transfer. For example, Lee and Ko investigated the effects of metformin on the gut microbiota using PICRUSt.[71] In this report, they used five groups of mice including those only on a high-fat diet, those on a high-fat diet and treated with metformin, those on a high-fat diet that transitioned to a normal diet, those on a normal diet and treated with metformin, and those that were only on a normal diet.[71] PICRUSt analysis identified that fatty acid metabolism of the gut microbiota was increased in the metformin-treated mice, which represents a potential explanation for the beneficial attributes of metformin therapy.[71] Unfortunately, this group did not validate the PICRUSt results with mass spectrometry-based metabolomics or targeted profiling to quantitate fatty acid levels.

Along with PICRUSt, metagenomic studies can also be run in conjunction with metabolomics to show, and confirm, bacterial functionality. HUMAnN2 (**H**MP **U**nified **M**etabolic **An**alysis **N**etwork 2) is an analytical pathway that can be used to analyze the metagenomics sequence results.[72] Like PICRUSt, HUMAnN2 investigates gut microbiome functionality. The major difference is that the HUMAnN2 pipeline uses metagenomic shotgun sequence reads from the Illumina Hiseq platform, whereas PICRUSt typically uses 16S rRNA gene sequencing reads from the Illumina Miseq. Whole genome sequencing involves sequencing bacterial DNA from a sample microbiome, whereas 16S rRNA gene sequencing only sequences the unique V4−V4 (or, depending on the application, other variable regions of the 16S rRNA gene) variable region of the bacterial genome. HUMAnN2 uses MetaPhlAn 2 (**Meta**genomic **Ph**ylogenetic **An**alysis 2) to assign taxonomy to the shotgun reads.[73] The HUMAnN2 algorithms

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use the MetaCyc database to assign pathways and their relative abundances to each sample. An earlier version of HUMAnN2 was used in a recent study of antibiotic resistant genes in the fecal microbiome.[74] It was reported that short-term exposure to clindamycin and ciprofloxacin had a lasting impact on the fecal microbiome by drastically decreasing the amount of butyrate producing bacteria.[74] The oral microbiome was much more resilient and had less antibiotic resistance pathways than the fecal microbiome after exposure to antibiotics.[74] Other metagenomic studies have been performed using different software packages other than HUMAnN2. One of these studies uses MOCAT (**M**etagen**o**mi**c** **A**nalysis **T**oolkit)[75] and CD-HIT[76] to investigate the effects of metformin on the gut microbiome.[77] The metagenomic study reported that metformin treatment caused an increase of *Escherichia* spp. in the gut which resulted in an increase of gas metabolism pathways, a common side effect of metformin.[77] While metagenomic studies can be time-consuming and computationally intensive, it remains an invaluable tool to help understand the functionality of the gut microbiota.

A recent framework for combining 16S rRNA sequencing, metagenomic data, and metabolomics data was described.[78] This framework associated metabolites with bacteria and monitored changes based on a disease state. While the concepts are indeed interesting, the incorporation of data from Illumina MiSeq or HiSeq would greatly enrich this approach and likely inform pharmacology and toxicology research. Developing new tools that integrate the vast and varied sources of data will likely provide incredibly informative and new perspectives by which to understand the impact of the gut microbiota on health and disease.

3. CONCLUSIONS AND PERSPECTIVE

It is clear that as the "forgotten organ"[79] the gut microbiota can significantly contribute to host metabolism in both beneficial and deleterious ways. Interest in the gut microbiota has been invigorated through technological advances in culture-independent sequence-based analysis of the microbiota as well as through mass spectrometry-based analysis and characterization of metabolites produced by or modified by the microbiota. As tools such as 16S rRNA gene sequencing, metagenomics, metatranscriptomics, and mass spectrometry-based metabolite profiling become commonplace, the integration of these different streams of data (Figure 2) will provide the most informative view of the microbiota's metabolic impact and function.[80,81] Further models based on genome scale models that combine in silico models and data collected from sequencing and metabolomics approaches are becoming more commonplace and likely will permit more generalized models to be developed and new hypotheses to be tested.[82,83] However, the bioinformatic tools necessary to handle and merge these data sets into a meaningful and comprehensive view of microbial metabolism remain an expanding and evolving area of science.

Tremendous efforts are currently underway to catalogue and characterize members of the human microbiome as part of the National Institutes of Health sponsored Human Microbiome Project.[84,85] The project has helped generate incredible insight into the microbiome of healthy and diseased humans and has established new resources (e.g., databases of reference genomes, sequence tools, protocols) to help accelerate as well as to standardize research in this area. Similarly, the National Institutes of Health Common Fund has established regional comprehensive metabolomics resource cores to increase accesibility to metabolite profiling services and resources.[86] As of yet, no formal integration of the two consortiums has been established, but as the fields progress, it seems likely that the microbiome and metabolomics research communities are destined for a long and productive relationship.

Lastly, it is important to consider not only the mechanisms by which the microbiota contribute to (or are themselves impacted by) drug metabolism but also how to incorporate this information when considering drug toxicity. This concept is even more important when considering how to develop therapeutics that target the microbiota and ultimately how to assess toxicity of the microbiota. Several recent examples suggest that known toxicants like the persistent organic pollutant and aryl hydrocarbon receptor activator, 2,3,7,8-tetrachlorodibenzofuran,[87] or heavy metal arsenic[88,89] can profoundly alter not only the gut microbiota community but also its metabolic function (e.g., changes in SCFA production). Futhermore, given that different disease states (e.g., obesity, diabetes) can impact the gut microbiota structure and function, how we will incorporate this information into drug toxicity assessment has yet to be determined. Clearly, biomarkers of microbome "health" and "toxicity" are required and will likely become important components of our overall understanding of drug and toxicant metabolism.

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Philip B. Smith is Director of Metabolomics at the Pennsylvania State University. Dr. Smith is an analytical chemist with extensive experience in the application of modern analytical chemistry techniques for the detection, identification, and quantitation of a wide variety of chemical and biochemical compounds. His work is focused on mass spectrometric applications, including the development and utilization of sensitive, compound-specific analytical methods; these methods often incorporate selective ionization and scanning techniques, resulting in the successful detection and identification of very low levels of compounds present in complex matrices.

Jeffrey M. Peters is Distinguished Professor and Associate Director of the Center for Molecular Toxicology and Carcinogenesis at the Pennsylvania State University. He has received a number of awards including The Society of Toxicology Achievement Award and The Huck Award for Outstanding Achievements in Life Sciences Research.

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Dr. Peters has performed seminal research focused on the biological role of the peroxisome proliferator-activated receptors (PPARs). His studies have provided clues to new molecular targets that may be suitable for the treatment and prevention of cancer.

Andrew D. Patterson is Associate Professor of Molecular Toxicology and Scientific Director of Metabolomics at the Pennsylvania State University. The Patterson lab studies the host metabolite−microbiome axis—specifically how the manipulation of gut bacteria through xenobiotic exposure impacts bile acid pools, their metabolism, and how they interact with host nuclear receptors. The lab employs a variety of cutting-edge tools including ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry-based metabolomics and conventional and gnotobiotic mouse models to facilitate its study of these pathways and understand their potential impact on human health and disease. A central question he and his lab are pursuing is how host nuclear receptors and the gut microbiota contribute to the development of nonalcoholic fatty liver disease.

■ ABBREVIATIONS

UHPLC-QTOFMS, ultra-high-pressure liquid chromatography coupled with quadrupole time-of-flight mass spectrometry; LC-MS, liquid chromatography coupled with mass spectrometry; GC-MS, gas chromatography coupled with mass spectrometry; MRM, mulitple reaction monitoring; NMR, nuclear magnetic resonance spectroscopy; SCFA, short chain fatty acid; PICRUSt, Phylogenic Investigation of Communities by Reconstruction of Unobserved States; INTERMAP, INTERnational collaborative study of MAcronutrients, micronutrients, and blood Pressure; MOCAT, MetagenOmiC Analysis Toolkit; MetaPhlAn, METAgenomic Phylogenetic Analysis; HUMAnN2, HMP Unified Metabolic Analysis Network; TMAO, trimethylamine-*N*-oxide; TMA, trimethylamine; FMO, flavin monooxygenase; MS^{n}, multistage mass spectrometry; 2,8-DHQ, 2,8-dihydroxyquinoline

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